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Gottlieb et al.
Cells Tissues Organs
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Thomson et al.
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2000

Nichols et al.
Experimental Cell Research
Vol. 215, No. 1
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1994

Wiles et al.
Experimental Cell Research
Vol. 247, No. 1
Pages 241-248
1999

Nichols et al.
Experimental Cell Research
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1994

Wiles et al.
Leukemia
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Pages 454-456
1997

Booth et al.
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Can Exogenous Stem Cells Be Used in Transplantation?

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Embryonic stem cells · Cell replacement therapy

Abstract

Today's most urgent problem in transplantation is the lack of suitable donor organs and tissues and as the population ages, demands for organs and tissue therapies will only increase. One alternative to organ transplantation is cell therapy whose aim is to replace, repair or enhance the biological function of damaged tissue or diseased organs. One goal of cellular transplantation thus has been to find a renewable source of cells that could be used in humans. Embryonic stem (ES) cells have the potential to proliferate in vitro in an undifferentiated and pluripotent state. Theoretically, ES cells are capable of unlimited proliferation in vitro. ES cells spontaneously differentiate into derivatives of all three primary germ layers: endoderm, ectoderm and mesoderm, hence providing cells in vitro which can theoretically be isolated and used for transplantation. Furthermore, these pluripotent stem cells can potentially be used to produce large numbers of cells that can be genetically modified in vitro. Once available, this source of cells may

obviate some of the critical needs for organ transplantation. Murine ES cells have been extensively studied and all available evidence indicates that all aforementioned expectations are indeed fulfilled by ES cells. ES cells as well as embryonic germ cells have recently been isolated and maintained in culture. The recent descriptions of human ES cells portend the eventual use of allogeneic in vitro differentiated cells for human therapy. This goal, however, is fraught with obstacles. Our aim is first to review the recent advances made with murine ES cells and then to point out potentials and difficulties associated with the use of human ES cells for transplantation.

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Abbreviations used in this paper

EG	embryonic germ cells
ES	embryonic stem cells
SCID	severe combined immune deficiency
tTA	tetracycline transactivator

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Introduction

Quiescent stem cells or combined progenitor cells allow organ or tissue self-repair; however, tissue damage or disease that disrupts cell function requires therapy in the form of transplantation or cell replacement. Today's most urgent problem in transplantation is the lack of suitable donor organs and tissues. In fact as few as 5% of the organs needed in the US will be available for those awaiting transplantation [Platt, 1998]. As the population ages, demands for organs and tissue therapies will only increase. One alternative to organ transplantation is cell therapy whose aim it is to replace, repair or enhance the biological function of damaged tissue or diseased organs [Gage, 1998]. One goal of cellular transplantation thus has been to find a renewable source of cells that could be used in humans.

Such an approach is potentially achievable by the transfer of isolated and characterized cells to a target organ in sufficient number and quality for them to survive and restore function. Sources of cells or tissues are self (autologous), same species (allogeneic), different species (xenographic), primary or immortalized cells (cell lines) and differentiated stem cells. The ability to cultivate, multiply and manipulate these cell types has either limited or encouraged their use in specific treatment protocols [Gage, 1998]. Where possible, autologous cells are preferential but frequently unavailable. The recent descriptions of human embryonic stem (ES) cells portend the eventual use of allogeneic in vitro-differentiated cells for human therapy. This goal is fraught with obstacles and will require integration of cell biology, immunology, tissue engineering, molecular biology, materials science, transplantation biology and the clinical expertise relevant to the disease being treated for any eventual intervention.

Recently an important first step towards reaching this goal has been accomplished by two laboratories, one of which has described the establishment of human ES cells and the other human embryonic germ (EG) cells [Shambloott et al., 1998; Thomson et al., 1998]. As cells in vitro can theoretically be isolated and used for transplantation, pluripotent stem cells can potentially be used to produce large numbers of cells that can be genetically modified in vitro. Once available, this source of cells may obviate some of the critical needs for organ transplantation. In order to appreciate the potential importance of these human ES or EG cells, it is worth examining the recent advances made with mouse ES cells.

Mouse ES Cells: Properties and Usefulness

Mouse ES cells have originally been derived from undifferentiated cells of the inner cell mass of preimplantation blastocysts or from eight-cell embryos [Doetschman et al., 1985, 1988]. These cells can proliferate in vitro in an undifferentiated, pluripotent state on a feeder layer of primary mouse embryonic fibroblasts or leukemia inhibitory factor-producing mouse fibroblastic cell lines (STO cells) [Williams et al., 1988]. Theoretically, ES cells are capable of unlimited proliferation in vitro. When injected into mice, ES cells can form teratomas that contain cell types derived from all three germ layers. Importantly, these cells in vitro can maintain a relatively normal and stable karyotype even with continual passaging. Upon removal of the mouse embryonic fibroblast feeder layer or more generally in the absence of leukemia inhibitory factor, ES cells spontaneously differentiate into derivatives of all three primary germ layers: endoderm, ectoderm and mesoderm [Evans and Kaufman, 1981; Martin, 1981; Wobus et al., 1984]. The in vitro differentiation of ES cells requires their initial aggregation to form structures, termed embryoid bodies, which then differentiate into a variety of specialized cell types, including cardiomyocytes (atrial, ventricular, pacemaker), smooth and skeletal muscle cells, hematopoietic cells, cartilage, melanocytes and neurons.

When returned to the embryonic environment by transfer into a host blastocyst or aggregation with blastomere stage embryos, ES cells behave like normal embryonic cells. They can contribute to all tissues in the resulting chimeras including gametes. ES cells thus have the full potential to develop along all lineages of the embryo proper. Importantly, genetic alterations introduced into ES cells can also be transmitted to the germline through the production of ES cell chimeras. Applying gene targeting technology to ES cells in culture allows genetic modification of endogenous genes and study of their function in vivo.

All these general properties explain the wide variety of applications for which ES cells have been instrumental.

Creation of New Animal Models

The existence of ES cells has allowed the creation, almost at will, of any kind of mutation in any given gene through the use of a wide variety of techniques which include selective homologous recombination and exon or promoter trap methods. So far, most animal models have been created as substitutes for human pathologies, and the use of ES cell technology in this context has only been

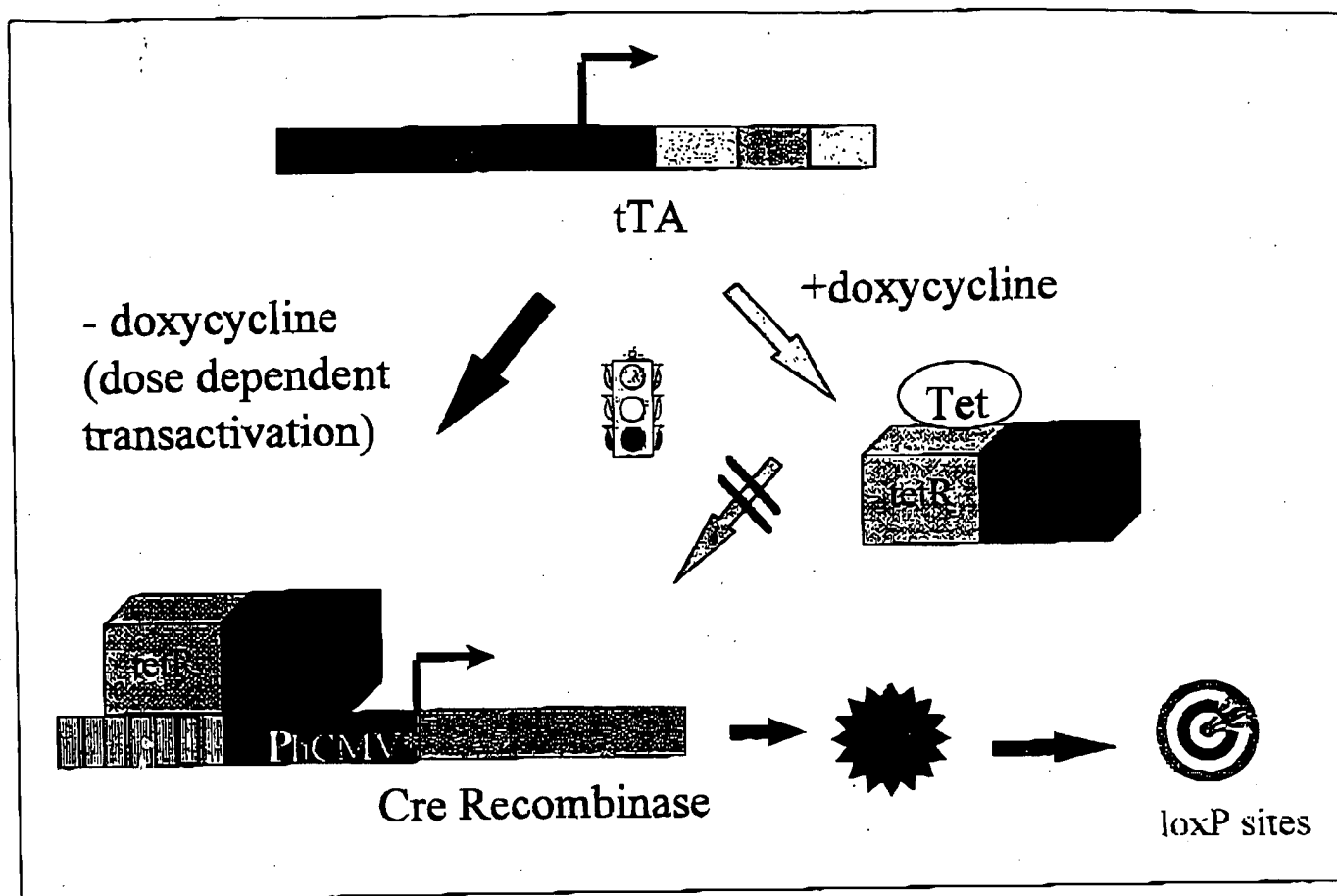


Fig. 1. Binary system for inducible Cre recombinase gene expression. In the binary system, tissue specificity is generated through use of a tissue-restricted promoter that regulates tTA gene transcription. The target for tTA is a minimal promoter fused downstream of seven operator sequences (TRE) of the *Escherichia coli* tet operon. Being bacterially derived, this gene is inactive in mammalian cells, unless

the tTA protein is expressed in the same cell. Temporal control over Cre recombinase transgene expression is achieved in the presence or absence of doxycycline. Use of the tTA system thus permits the study of gene expression in specific ES-derived cells or mouse tissues during specific developmental stages.

successfully applied to mice. One such example is provided by the mouse model in which the cardiac myosin heavy chain has been mutated (Arg⁴⁰³→Gln) in order to reproduce familial hypertrophic cardiomyopathy [Geisterfer-Lowrance et al., 1996]. Many of these models were somewhat disappointing mostly because pathological manifestations are the end result of an array of secondary effects which may be different between humans and mice.

Another type of animal model using ES cells has allowed the creation of animals in which large-scale chromosome alterations have been engineered. Two such models were recently described [Smith et al., 1995; Van Deursen et al., 1995] which take advantage of the Cre-

loxP system. Very briefly, loxP sites are introduced at specific sites in the genome, on different chromosomes, and the cells, that carry these sites, are further transfected by a Cre recombinase-carrying vector. As a very rare event, the Cre recombinase will induce a recombination between the two sites, hence leading to a chromosomal translocation. Smith and his colleagues targeted the translocation between the myc gene (chromosome 15) and immunoglobulin heavy chain genes (chromosome 12) while Van Deursen and his colleagues obtained a translocation between the Dek gene (chromosome 13) and the Can gene (chromosome 2). Such a strategy should allow the design of a variety of chromosome rearrangements specific to different types of cancer or of various pathologies.

Cre recombinase and loxP sites can also be used in a binary system to temporally and/or spatially regulate gene expression. Although several inducible systems exist [Kuhn et al., 1995; Logie and Stewart, 1995], perhaps the best regulatory system of this sort relies on tetracycline regulation as developed by Bujard [Gossen and Bujard, 1992]. When placed under the control of a lineage-specific promoter (e.g., α -myosin heavy chain promoter in heart) tissue-restricted expression of the tetracycline transactivator (tTA) can be achieved [Yu et al., 1996; Redfern et al., 1999]. In this system, doxycycline administered in the mouse diet binds to the tTA, preventing activation of the tetO promoter, whereas in the reverse tTA system, transcriptional activation is induced by addition of doxycycline. In the binary system, inducible control of Cre recombinase would be regulated by binding of the tTA to the tetO promoter (fig. 1). Under conditions where the tetO promoter is not activated, sequences containing the loxP site should remain intact; however, tTA transactivation by withdrawal of doxycycline would be expected to bind to the tetO promoter and drive expression of the Cre recombinase. Available loxP sites would then be targeted. The advantage of such a system rests in its ability to induce the excision of a critical exon in the target gene flanked by loxP sites at specific developmental stages (embryonic, fetal, perinatal adult). Such a system has already been proven to be functional in mice [Mayford et al., 1996; Tsien et al., 1996].

In vitro Studies

Approaches such as those described for making animal models require prior knowledge of the sequence and genomic organization of each gene to be studied. The development of ES cells and their study in vitro has, however, provided a novel approach to study development or study the effects of mutations in the genome before their introduction into the mouse germline. For example, differentiation of ES cells and formation of embryoid bodies have provided a major opportunity to study the regulation of lineage commitment. This approach has led to the identification of novel precursor cells [Choi et al., 1998] and has allowed a better characterization of specific pathways such as the expression of myogenic factors during skeletal muscle differentiation [Braun and Arnold, 1996]. Additionally, ES cells have been used as a complementary system to study promoter elements instead of using transgenic animals [Pari et al., 1991]. For example, the random insertion of exogenous DNA into single sites in the mammalian genome (gene trapping) provides a genome-wide strategy for functional genomics. When performed in tan-

dem with the developmental potential of ES cells to differentiate into distinct cell lineages (expression trapping), such techniques are useful for the identification of novel genes expressed in developing systems. Stanford et al. [1998] successfully used this system to identify several novel hematopoietic and vascular genes.

ES cells have also been used to study the function of genes. In particular the system is well suited to understand the role of genes through 'loss of function' studies by developing ES cell lines that lack a specific gene. This is of particular interest when the inactivation of the gene is lethal in the animal. Such an example was recently published in the case of ES cells lacking the β_1 integrin [Rohwedel et al., 1998; Wobus and Guan, 1998]. Recently, we successfully prepared several neomycin-resistant R1 ES cell clones containing exons for the cardiac calcium release channel flanked by loxP sites. Knockout of this gene results in embryonic lethality, but in the context of this recombination event, flanking of the exon by loxP sites should not have affected gene expression. To ensure that these ES cell clones acted similarly to the parental cells before injection into mouse blastocysts, the undifferentiated ES cell clones were differentiated in vitro [Maltsev et al., 1994]. Clonal ES cells were trypsinized, and hanging drops were prepared with 600 or 800 ES cells/20 μ l of DMEM media containing 20% FBS and additives. Following 2 days in a hanging drop, the developing embryoid body was transferred to suspension culture with maintenance media (DMEM/15% FBS/additives). After 4–5 more days in suspension culture, the embryoid bodies were plated and allowed to attach. Within 1–5 days, >90% of the plated embryoid bodies from both the parental and clonal ES cell lines contained spontaneously and rhythmically contracting cardiomyocytes (unpubl. results). The results suggested that modifications in the introns of this gene had not adversely affected its expression after homologous recombination. Conversely, ES cells have also been an ideal in vitro system for 'gain of function' studies, since they permit the study of specific gene overexpression on a given differentiation lineage without having to worry about the effects of such an overexpression on the overall embryonic development [Grépin et al., 1997].

Cell Therapies

Well-defined differentiation protocols from mouse ES cells have been developed for neuronal cells [Gottlieb and Huettner, 1999], adipocytes [Dani, 1999], smooth muscle myocytes [Drab et al., 1997], skeletal muscle myocytes and hematopoietic cells [Wiles and Keller, 1991]. A num-

ber of protocols have also been described for the production and isolation of cardiomyocytes [Wobus et al., 1991]. Spontaneously and rhythmically contracting cardiomyocytes for example can be isolated by mechanical dissection and collagenase treatment. Individual cells can then be used in single cell assays. ES-derived cardiomyocytes express α - and β -myosin heavy chain, α -tropomyosin, myosin light chain-2v, atrial natriuretic factor, phospholamban, L-type calcium channels [Hescheler et al., 1997] SR CaATPase (unpubl. data), ryanodine receptor type 2 (unpubl. data), and they show normal contractile sensitivity to calcium. Electrophysiological analyses have identified action potentials typical for atrial, ventricular and conduction system cardiomyocytes. The cells in vitro do not, however, seem to form T tubules or show several other characteristics of adult cardiomyocytes.

Even when optimal conditions are used to induce differentiation, the percentage of differentiated cells belonging to one phenotype still remains very small (in the case of cardiac differentiation, this percentage is frequently of the order of 3–5%). Therefore, one of the major problems which arises is: how can one isolate a pure population of ES cell-derived differentiated cells that could potentially be used for transplantation?

Several groups have been successful in isolating individual cell types following genetic manipulation and in vitro differentiation of ES cells. Dinsmore et al. [1996] reported the differentiation of ES cells into nearly homogeneous populations of neurons and skeletal muscle cells. For skeletal muscle cell isolation, ES cells were stably transfected with a gene coding for the muscle-specific regulatory factor MyoD. When induced with DMSO, the ES cells expressing high levels of MyoD differentiated into skeletal myoblasts that fused and formed myotubes capable of contraction. Greater than 90% of those cells expressing high levels of MyoD were found to be of skeletal muscle origin [Dekel et al., 1992]. Li et al. [1998] described a system which allowed for the efficient purification of neuroepithelial progenitor cells. Specifically, they targeted a promoterless neomycin cassette to the SOX2 gene, so that only those cells expressing SOX2 following in vitro differentiation would be neomycin (G418)-resistant. The SOX2 gene encodes for a DNA binding protein whose expression is restricted to the early neural plate. The neomycin-resistant cells were shown to differentiate into neuronal networks in the absence of other cell types.

Field and his group have successfully shown that genetically selected cardiomyocytes can be isolated from differentiating ES cells and could thereafter be used to form

stable intracardiac grafts [Klug et al., 1996]. This group prepared an ES cell line carrying a fusion gene comprised of the cardiac α -myosin heavy chain promoter and sequences encoding aminoglycoside phosphotransferase to confer neomycin (G418) resistance. Following stable transfection, they were able to select a relatively pure population of cardiomyocytes (99.6%) and inject them into the ventricular myocardium of mdx mice. Subsequent analyses indicated the presence of ES-derived cardiomyocyte grafts for at least 7 weeks after implantation.

Lastly it is also possible to isolate ES-derived differentiated cells without having to rely on any selection procedure but through the use of the specific expression of a fluorophore by the differentiated cells. Such an example was shown in the case of cardiac differentiation where ES cells were stably transfected by the gene encoding the green fluorescent protein under the control of the cardiac α -actin promoter [Kolossov et al., 1998]. Upon differentiation only cardiomyocytes express the green fluorescent protein and it is possible to isolate them by flow-cytometric methods.

All these data show that specific and relatively pure cell types can be isolated from in vitro differentiated ES cells. Whether similar success will be forthcoming from the human ES cells is unknown, but the prospects seem very favorable.

Human ES Cells: ES and EG Cells

In November 1998, Thomson et al. [1998] reported the derivation of the first ES cell lines from human blastocysts. Several characteristics of ES cells were determined including a high ratio of nucleus to cytoplasm, prominent nucleoli, and colony morphology similar to that of ES cells derived from rhesus monkey (table 1). One of the ES cell lines isolated retained a normal XX karyotype after 6 months of culture. This human ES cell line expressed high levels of telomerase activity and expressed cell surface markers characteristic of undifferentiated nonhuman primate ES and human embryonic carcinoma cells. The undifferentiated cells showed positive alkaline phosphatase activity. Upon injection into severe combined immunodeficient (SCID) beige mice, teratomas were formed and derivatives of all three germ layers identified. With these tests, the evidence is very good that human ES cells have in fact been derived.

Shamblott et al. [1998] subsequently reported the derivation of pluripotent stem cells from cultured human primordial germ cells. Although examined to a lesser extent

Table 1. Characteristics of pluripotent embryonic cell lines: mouse versus human

	Mouse ES cells	Human ES cells	Human EG cells
<i>Undifferentiated</i>			
Unlimited, undifferentiated proliferation	Yes	Probable	Possible
Compact, multilayered colonies	Yes	Yes	Yes
High nuclear to cytoplasmic ratio	Yes	Yes	Yes
Alkaline phosphatase activity	Yes	Yes	Yes
High levels of telomerase activity	Yes	Yes	Not tested
Stable developmental potential	Yes	Yes	Possible
<i>Differentiated</i>			
Potential for in vitro differentiation	Yes	Yes	Yes
Express conserved epitope markers	Yes	Yes	Yes
Ability to give rise to multiple cell types	Yes	Yes	Yes
Ability to contribute to germline	Yes	Unknown/ unethical	Unknown/ unethical

than the ES cells described by Thomson et al., these cells also showed many of the characteristics of pluripotency: high levels of AP activity, compacted multicellular colonies, positive staining for a number of appropriate germ-line markers, and maintenance of normal karyotypic features. The immortality of these cells remains to be demonstrated.

Potential Developments for Human ES/EG Cells

The derivation of these stem cells represents the first major step in obtaining a renewable, tissue culture source of human cells capable of differentiating into a wide variety of cell types. The implications that relate to the development of these cells have broad applications for basic research and potential clinical use in humans. These pluripotent cells will be useful for in vitro studies of normal human embryogenesis, abnormal development (generation of cell lines with modified genes and chromosomes), human gene discovery, and drug and teratogen testing.

Use in Basic Research

Their acquisition also bodes well for basic science investigations. Particularly relevant will be the study of development and their use in vitro to understand fundamental biological problems. As already mentioned, the availability of mouse ES cells has generated an enormous amount of information on the developmental programs associated with lineage commitment, on gene function and regulation of gene expression. The same types of studies should now be possible with human ES cells.

Cell Replacement Therapies

Cultivation in vitro and isolation of specific cell types should lead to their use as a renewable source of cells for tissue transplantation, cell replacement, and gene therapies. The group of Field has proven this concept experimentally in mouse heart not only with modified ES cells as described above, but with C2C12 myoblast and fetal cardiomyocyte grafts [Koh et al., 1993, 1995]. Exploitation of the newly derived human ES cells may provide a renewable and potentially unlimited source for cell replacement in humans. This latter application could eventually preclude the direct use of fetal tissue in transplantation therapies. Clinical targets might include neurodegenerative disorders, diabetes, spinal cord injury, and hematopoietic repopulation and myocyte grafting. However, without even thinking of using these stem cells for transplantation therapy in humans, an exciting potential that they offer is the possibility to create animal models allowing investigators to study the fate of human cells. A number of studies have shown that it is possible to graft human cells in SCID mice and thus, provided that stem cells are available, it could be possible to create an animal in which a tissue (or part of it) is human. Such examples have already been described in the case of bone marrow-derived cells [Greiner et al., 1998]. Animals of that sort should prove to be very instrumental in the analysis of both normal and pathological development. An example of the potential of this approach has recently been published in the case of the McCune-Albright syndrome [Bianco et al., 1998]. Since ES cells can generate a wide variety of precursor cells it should, in principle, be possible

ble to generate mice carrying the corresponding human tissues. Furthermore, since the ES cells can be modified, the available precursor cells should be both normal and pathological.

Limitations and Problems

Before the transfer of any ES-derived cells to humans can be accomplished, a number of experimental obstacles must be overcome and theoretical questions addressed. Any cells used in transplantation would need to be autologous/immunologically masked, expandable in vitro, show appropriate integration of selection vectors into either targeted or inconsequential random genomic sites, and have homogeneous cell phenotypes. Pure populations of cell types would be a prerequisite since introduction of contaminating pluripotent ES cells during transplantation might give rise to teratomas. In the scenario where human ES-derived differentiated cells – say cardiomyocytes, neuronal cells, hematopoietic cells or vascular smooth muscle cells – could be isolated, appropriate steps would need to be taken to prevent rejection of the transplanted cells. A first step towards this goal would be the establishment of protocols to isolate pure cell populations similar to what has been done with mouse ES cells in culture (see above).

In transplantation scenarios, prevention of rejection is one of the paramount issues as all stromal-derived and hematopoietic-derived (except RBCs) cells express either HLA class I and/or class II molecules which can be recognized by specific lymphocytes in a foreign host. There are many regimens utilized to immunosuppress hosts prior to transplant to facilitate graft acceptance. They include immunosuppressive therapy (i.e. cyclosporin A, Cyclosporin), systemic tolerance to foreign HLA and immunotherapy to destroy graft-reactive lymphocytes. Only animals or humans with similar MHC/HLA molecules could be donors for other hosts. Practically this would require determination of allogeneic compatibility. It is quite easy to define the MHC (rodents) or HLA (humans) backgrounds. There are PCR-based systems to define human class I and class II expression as well as many antibodies for flow cytometry and immunohistological analysis. In rodents, it is much easier as many rodent species have already been defined so it would be simple to predict MHC differences and predict reactivities. For ES cells derived from one human individual, all the HLA molecules would be clonal. As such, banks of ES cells with known HLA backgrounds could be established. When subjected to appropriate genetic manipulation, specific cell types could be isolated; however, the need to isolate multiple pure populations of cells with defined HLA mol-

ecules represents an enormous amount of work. Perhaps in concert with the binary system described above, it should be possible to identify loci in the human genome where specific loxP sites could be inserted without deleterious functional consequences. Once prepared, it should be possible to use these loxP sites in conjunction with Cre recombinase to insert almost any vector construct into the human genome, permitting a rapid procedure for selecting pure populations.

Would it be possible to prepare 'universal donor cells' derived from ES cells? Theoretically, it could be possible if all class I and class II molecules were eliminated from the cell surface of ES cells. This would require extensive gene targeting and early attempts would probably have to be directed against class I molecules. The consequences of such extensive gene targeting are, however, difficult to assess. Finally, it would be necessary to prevent karyotypic changes during passaging and preparation of ES-derived cells. This is no small undertaking for even though cells may show normal karyotypes, there is no assurance that consequential changes in the genome have not occurred. As with nuclear transfer technology in the production of Dolly, it was thought that her genome would carry the mutations that had accumulated prior to nuclear transfer. Dolly is, however, still alive and her offspring seem perfectly normal. Perhaps this bodes well for the grafting of genetically modified material that has been passaged in tissue culture for very long periods of time, but the potential for genetic mutations that may have serious consequences cannot be discounted. The increased incidence of the large offspring syndrome from the cattle and sheep industry indicates some of the potential dangers of epigenetic phenomena associated with the preparation of cells in vitro for transfer to blastocysts [Young et al., 1998]. Perhaps such scenarios will not be of great consequence with sufficiently differentiated and selected cells; however, other techniques or rapid screening procedures (e.g. use of chip technology) may be necessary to control the quality of the cells introduced into a transplantation host.

Conclusions.

The ES cell technology has revolutionized modern biology and has provided us with an unprecedented opportunity to understand normal development and to gain new insights into the mechanisms that lead to pathological situations. Mouse ES cells have provided the means to study development, gene expression and create

models to look at the effects of specifically modified genes on mouse phenotype. Spatial and temporal control of gene expression in the mouse genome should permit better elucidation of the exact consequences resulting from altered gene expression/function in the absence of developmental compensation (as seen with traditional knock-out techniques). The development of human ES cells is a major step towards the possibility of applying this technology to the treatment of human diseases. Although it is unclear if human ES cells will be as experimentally robust as mouse ES cells, in the event that human ES cells can be genetically modified and specific cell types isolated, their

use in transplantation studies may become reality. A renewable source of human cells is the goal; however, much remains to be accomplished before in vitro-differentiated human ES cells can be used therapeutically.

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References

- Bianco, P., S.A. Kuznetsov, M. Riminucci, L.W. Fisher, A.M. Spiegel, P.G. Robey (1998) Reproduction of human fibrous dysplasia of bone in immunocompromised mice by transplanted mosaics of normal and Gsa-mutated skeletal progenitor cells. *J Clin Invest* 101: 1737-1744.
- Braun, T., H.-H. Arnold (1996) Myf5 and myoD are activated in distinct mesenchymal stem cells and determine different skeletal muscle cell lineage. *EMBO J* 15: 310-318.
- Choi, K., M. Kennedy, A. Kazarov, J.C. Papadimitriou, G. Keller (1998) A common precursor for hematopoietic and endothelial cells. *Development* 125: 725-732.
- Dekel, L., Y. Magal, S. Pearson-White, C.P. Emerson, M. Shani (1992) Conditional conversion of ES cells to skeletal muscle by an exogenous myoD1 gene. *New Biol* 4: 217-224.
- Dani, C. (1999) Embryonic stem cell-derived adipogenesis. *Cells Tissues Organs* 165: 173-180.
- Dinsmore, J., J. Ratliff, T. Deacon, P. Pakzaban, V. Jacoby, W. Galpern, V. Isacson (1996) Embryonic stem cells differentiated in vitro as a novel source of cells for transplantation. *Cell Transplant* 5: 131-143.
- Doetschman, T., P. Williams, N. Macda (1988) Establishment of hamster blastocyst-derived embryonic stem (ES) cells. *Dev Biol* 127: 224-227.
- Doetschman, T.C., H. Eistetter, M. Katz, W. Schmidt, R. Kemler (1985) The in vitro development of blastocyst-derived embryonic stem cell lines: Formation of visceral yolk sac, blood islands and myocardium. *J Embryol Exp Morphol* 87: 27-45.
- Drab, M., H. Haller, R. Bychkov, B. Erdmann, C. Lindschau, H. Haase, I. Morano, F.C. Luft, A.M. Wobus (1997) From totipotent embryonic stem cells to spontaneously contracting smooth muscle cells: A retinoic acid and dbcAMP in vitro differentiation model. *FASEB J* 11: 905-915.
- Evans, M.J., M.H. Kaufman (1981) Establishment in culture of pluripotent cells from mouse embryos. *Nature* 292: 154-156.
- Gage, F.H. (1998) Cell therapy. *Nature* 392: 18-24.
- Geisterfer-Lowrance, A.A.T., M. Christe, D.A. Conner, J.S. Ingwall, F.J. Schoen, C.E. Seidman, J.G. Seidman (1996) A mouse model of familial hypertrophic cardiomyopathy. *Science* 272: 731-734.
- Gossen, M., H. Bujard (1992) Tight control of gene expression in mammalian cells by tetracycline-responsive promoters. *Proc Natl Acad Sci USA* 89: 5547-5551.
- Gottlieb, D.I., J.E. Huettner (1999) An in vitro pathway from embryonic stem cells to neurons and glia. *Cells Tissues Organs* 165: 165-172.
- Greiner, D.L., R.A. Hesselton, L.D. Shultz (1998) SCID mouse models of human stem cell engraftment. *Stem Cells* 16: 166-177.
- Grépin, C., G. Nemer, M. Nemer (1997) Enhanced cardiogenesis in embryonic stem cells overexpressing the GATA-4 transcription factor. *Development* 124: 2387-2395.
- Hescheler, J., B.K. Fleischmann, S. Lentini, V.A. Maltsev, J. Rohwedel, A.M. Wobus, K. Addicks (1997) Embryonic stem cells: A model to study structural and functional properties in cardiomyogenesis. *Cardiovasc Res* 36: 149-162.
- Klug, M.G., M.H. Soonpaa, G.Y. Koh, L.J. Field (1996) Genetically selected cardiomyocytes from differentiating embryonic stem cells form stable intracardiac grafts. *J Clin Invest* 98: 216-224.
- Koh, G.Y., M.G. Klug, M.H. Soonpaa, L.J. Field (1993) Differentiation and long-term survival of C2C12 myoblast grafts in heart. *J Clin Invest* 92: 1548-1554.
- Koh, G.Y., M.H. Soonpaa, M.G. Klug, H.P. Pride, B.J. Cooper, D.P. Zipes, L.J. Field (1995) Stable fetal cardiomyocyte grafts in the hearts of dystrophic mice and dogs. *J Clin Invest* 96: 2034-2042.
- Kolossov, E., B.K. Fleischmann, Q. Liu, W. Bloch, S. Vintchenko-Karpinski, O. Manzke, G.J. Li, H. Bohlen, K. Addicks, J. Hescheler (1998) Functional characteristics of ES cell-derived cardiac precursor cells identified by tissue-specific expression of the green fluorescent protein. *J Cell Biol* 143: 2045-2056.
- Kuhn, R., F. Schwenk, M. Aguet, K. Rjowsky (1995) Inducible gene targeting in mice. *Science* 269: 1427-1429.
- Li, M., L. Pevny, R. Lovell-Badge, A. Smith (1998) Generation of purified neural precursors from embryonic stem cells by lineage selection. *Curr Biol* 8: 971-974.
- Logie, C., A.F. Stewart (1995) Ligand-regulated site-specific recombination. *Proc Natl Acad Sci USA* 92: 5940-5944.
- Maltsev, V.A., A.M. Wobus, J. Rohwedel, M. Badier, J. Hescheler (1994) Cardiomyocytes differentiated in vitro from embryonic stem cells developmentally express cardiac-specific genes and ionic currents. *Circ Res* 75: 233-244.
- Martin, G.R. (1981) Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells. *Proc Natl Acad Sci USA* 78: 7634-7638.
- Mayford, M., M.E. Bach, Y.-Y. Huang, L. Lang, R.D. Hawkins, E.R. Kandel (1996) Control of memory formation through regulated expression of a CaMKII transgene. *Science* 274: 1678-1683.
- Pari, G., K. Jardine, M.W. McBurney (1991) Multiple CARG boxes in the human cardiac actin gene promoter required for expression in embryonic cardiac muscle cells developing in vitro from embryonal carcinoma cells. *Mol Cell Biol* 11: 4796-4803.
- Platt, J.L. (1998) New directions for organ transplantation. *Nature* 392: 11-17.
- Redfern, C.H., P. Coward, M.Y. Degtyarev, E.K. Lee, A.T. Kwa, L. Hennighausen, H. Bujard, G.I. Fishman, B.R. Conklin (1999) Conditional expression and signaling of a specifically designed Gi-coupled receptor in transgenic mice. *Nat Biotechnol* 17: 165-169.

- Rohwedel, J., K. Guan, W. Züschratter, S. Jin, G. Ahnert-Hilger, D. Fürst, R. Fässler, A.M. Wobus (1998) Loss of $\beta 1$ integrin function results in a retardation of myogenic but an acceleration of neuronal differentiation of embryonic stem cells in vitro. *Dev Biol* 201: 167-184.
- Shenbrot, M.J., J. Axelman, S. Wang, E.M. Bugg, J.W. Littlefield, P.J. Donovan, P.D. Blumenthal, G.R. Huggins, J.D. Gearhart (1998) Derivation of pluripotent stem cells from cultured human primordial germ cells. *Proc Natl Acad Sci USA* 95: 13726-13731.
- Smith, A.J.H., M.A.D. Sousa, B. Kwabi-Addo, A. Heppell-Parton, H. Impey, P. Rabbitts (1995) A site-directed chromosomal translocation induced in embryonic stem cells by Cre-loxP recombination. *Nat Genet* 9: 376-385.
- Stanford, W.L., G. Caruana, K.A. Vallis, M. Inamdar, M. Hidaka, V.O. Butch, A. Bernstein (1998) Expression trapping: Identification of novel genes expressed in hematopoietic and endothelial lineages by gene trapping in ES cells. *Blood* 92: 4622-4631.
- Thomson, J.A., J. Itskovitz-Eldor, S.S. Shapiro, M.A. Waknitz, J.J. Swiergiel, V.S. Marshall, J.M. Jones (1998) Embryonic stem cell lines derived from human blastocysts. *Science* 282: 1145-1147.
- Tsien J.Z., D.F. Chen, D. Gerber, C. Tom, E.H. Mercer, D.J. Anderson, M. Mayford, E.R. Kandel, S. Tonegawa (1996) Subregion- and cell type-restricted gene knockout in mouse brain. *Cell* 87: 1317-1326.
- Van Deursen, J., M. Fornerod, B. Van Roes, G. Grosveld (1995) Cre-mediated site-specific translocation between nonhomologous mouse chromosome. *Proc Natl Acad Sci USA* 92: 7375-7380.
- Wiles, M.V., G. Keller (1991) Multiple hematopoietic lineages develop from embryonic stem (ES) cells in culture. *Development* 111: 259-267.
- Williams, R.L., D.J. Hilton, S. Pearce, T.A. Willson, C.L. Stewart, D.P. Gearing, E.F. Wagner, D. Metcalf, N.A. Nicola, N.M. Gough (1988) Myeloid leukemia inhibitory factor maintains the developmental potential of embryonic stem cells. *Nature* 336: 684-687.
- Wobus, A.M., K. Guan (1998) Embryonic stem cell-derived cardiac differentiation: Modulation of differentiation and 'loss-of-function' analysis in vitro. *Trends Cardiovasc Med* 8: 64-74.
- Wobus, A.M., H. Holzhausen, P. Jäkel, J. Schöneich (1984) Characterization of a pluripotent stem cell line derived from a mouse embryo. *Exp Cell Res* 152: 212-219.
- Wobus, A.M., G. Wallukat, J. Hescheler (1991) Pluripotent mouse embryonic stem cells are able to differentiate into cardiomyocytes expressing chronotropic responses to adrenergic and cholinergic agents and Ca^{2+} channel blockers. *Differentiation* 48: 173-182.
- Young, L.F., K.D. Sinclair, I. Vilmot (1998) Large offspring syndrome in cattle and sheep. *Rev Reprod* 3: 155-163.
- Yu, Z., C.S. Redfern, G.J. Fishman (1996) Conditional transgene expression in the heart. *Circ Res* 79: 691-697.